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Overexpression of transglutaminase 2 accelerates the erythroid differentiation of human chronic myelogenous leukemia K562 cell line through PI3K/Akt signaling pathway

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Abstract Transglutaminase 2 (TG2) is a GTP-binding protein with transglutaminase activity. Despite advances in the characterization of TG2 functions and their impact on cellular processes, the role of TG2 in Human chronic myelogenous leukemia K562 cell line is still poorly understood. To understand the biological significance of TG2 during the differentiation of K562 cells, we established and characterized K562 cells that specifically express TG2. Non-transfected K562 cells showed the increase of membrane-bound-TG2 level after 3 days in the response to Hemin and all *trans*-retinoic acid (tRA), indicating that membrane recruitment of TG2 is occurred during the erythroid differentiation. However, membrane recruitment of TG2 in TG2-transfected cells revealed within earlier time period, compared with that in vector-transfected cells. The ability of membrane-bound-TG2 to be photoaffinity-labeled with [α-^{32}P]GTP was also increased in TG2-transfected cells. TG2-transfected cells activated Akt phosphorylation and inactivated ERK1/2 phosphorylation, compared with vector-transfected cells. Furthermore, phosphorylation of CREB, one of the Akt substrates, was increased in TG2-transfected cells and this phenomenon was confirmed by RT-PCR analysis of several marker genes related with erythroid lineage in the absence of PI3K specific inhibitor, Wortmannin, indicating that PI3K/Akt signaling pathway also involved in the differentiation of the cell. Finally, as results of benzidine positive staining as well as hemoglobinization analysis, overexpression of TG2 revealed acceleration of the erythroid differentiation of K562 cells. Taken together, there was no increased TG2 expression level in the response of Hemin/tRA and delayed differentiation in vector transfected cells than in TG2-transfected cells, suggesting that suppression of TG2 expression may retard the erythroid differentiation of K562 cells. Therefore, our study may give a new insight for another aspect of the development of this disease.

Keywords: Transglutaminase 2; GTP-binding protein; Hemin/tRA; Erythroid differentiation; Human chronic myelogenous leukemia K562 cell line; PI3K/Akt signaling pathway

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Abbreviations: TG2, transglutaminase 2; CML, chronic myelogenous leukemia; tRA, all *trans*-retinoic acid; CREB, cyclic-AMP response element binding protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FBS, fetal bovine serum; RT-PCR, reverse transcriptase-polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate

1. Introduction

Transglutaminase 2 (TG2) is the most ubiquitously expressed member of the transglutaminase family of proteins [1]. TG2 exhibits at least two distinct enzymatic activities. One is a Ca^{2+}-dependent transamidating activity that cross-links proteins or incorporates polyamines into protein substrates [2,3] and the other is GTPase (Gh) functioning as a GTP-binding protein with transglutaminase activity and receptor signaling function [4]. The most frequently reported cellular outcome associated with aberrant TG2 transamidation activity has been the induction of apoptosis [5–9]. However, several studies provide evidences that TG2 has been implicated in regulating cell differentiation [10–12] and cell survival [13,14]. Therefore, TG2 is a multi-functional enzyme that can modulate several biological events.

Cell differentiation is a complex process regulated by an interplay among intrinsic cellular programs, cell–cell and cell–substrate interactions, and a plethora of soluble extracellular signaling molecules, including hormones, growth factors, cytokines, trophic factors, and morphogens. All *trans*-retinoic acid (tRA), the biologically active form of vitamin A, plays a prominent role in regulating the transition from the proliferating precursor cell to the postmitotic differentiated cell, and there are many examples in the literature of distinct cell types whose differentiation is under the control of tRA [15–19]. However, no TG2 was induced following exposure to tRA in human chronic myelogenous leukemia K562 cell line [20], although it has been well established that tRA induced TG2 expression through the upregulation of the transcriptional activities of its receptors [21].

K562 cell line was established from a patient with chronic myeloid leukemia (CML) in terminal blast crisis [22] and this cell line can be induced to undergo erythroid differentiation by various compounds, including hemin [23] and megakaryocytic differentiation by phorbol 12-myristate 13-acetate (PMA). It has also been reported that retinoids (retinoic acid and synthetic retinoids) dose-dependently enhance hemin-induced erythroid differentiation of K562 cells [24].

Despite advances in the characterization of TG2 functions and its impact on cellular processes, the role of TG2 during the differentiation of K562 cells is still poorly understood. We demonstrate the hypothesis that TG2 recruitment into membrane is essential for the process of cellular differentiation of K562 cells into erythroid lineage.

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2. Materials and methods

2.1. Cell culture

The human CML K562 cell line was used for stable transfection with TG2 cDNA (gift from Mie-Jae Im, Chunbuk Univ. Korea) using LipofectAMINE reagent following the manufacturer's instructions. All cell lines were maintained using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin G (Life Technologies) at 37 °C in a humidified 5% CO_2 incubator. Transfected cells were then selected and maintained by cell culture medium containing 600 μg/ml of G418 sulfate.

Hemin and all tRA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). To induce erythroid differentiation, K562 cells were cultured for different times in the presence of 2.5 nM hemin/1 μM tRA. To induce megakaryocytic differentiation, the cells were treated with phorbol 12-myristate 13-acetate (PMA, 10 nM).

2.2. Western blot analysis

K562 cells were lysed in RIPA buffer containing 150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 2 mM EDTA, 10% (v/v) glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM Na_3VO_4, 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 kallikrein inhibitor units of aprotinin per ml. Cell lysates were clarified by centrifugation at $14\,000 \times g$ for 10 min at 4 °C. Proteins were separated on 10% polyacrylamide gels and then transferred to nitrocellulose (NC) membrane. After blocking non-specific bindings, the NC membranes were incubated with specific antibodies, anti-phospho-Akt (SantaCruz, USA), anti-phospho-ERK1/2 and anti-ERK1/2 (SantaCruz, USA), anti-phospho-p38 MAPK (Cell Signaling, USA), anti-phospho-JNK (Cell Signaling, USA), anti-TG2 (CUB 7402, Biomeda, USA), anti-phospho-CREB (Upstate, USA) and anti-GAPDH (Chemicon, USA). After washing the membranes with PBS three times, they were further incubated with horseradish peroxidase-conjugated antibody. Immunoblots were revealed by autoradiography using the enhanced chemiluminescence detection kit (Amersham Biosciences, UK). Protein content of the samples was determined by the Bradford method [25] using the Bio-Rad protein assay reagent.

2.3. Preparation of membrane proteins

The cells were washed with PBS three times and lysed with buffer containing 150 mM NaCl, 20 mM Tris (pH 7.5), 2 mM EDTA, 10% (v/v) glycerol, 1 mM Na_3VO_4, 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 kallikrein inhibitor units of aprotinin per ml, and total membranes collected by centrifugation (13 000 rpm for 10 min). The membrane proteins were solubilized with RIPA buffer and collected by centrifugation (13 000 rpm for 15 min).

2.4. Immunoprecipitation

Samples were pre-cleared with 50 μl of protein A-agarose beads at 4 °C for 1 h and clarified by centrifugation at 14 000 rpm for 10 min. The precleared lysate was incubated with an antibody for 1 h, then 50 μl of protein A-agarose beads was added and the mixture was further incubated for 1 h. After extensive washing with RIPA buffer, the immunoprecipitated proteins eluted from beads were subjected to 10% SDS–PAGE under reducing condition.

2.5. GTP binding assay

For photoaffinity labeling, membrane proteins (1 mg/ml) from each cell line were incubated with 0.1 mCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$ at room temperature for 20 min, transferred to an ice-bath, and cross-linked for 8 min by UV irradiation [26]. GTP-bound TG2 was then immunoprecipitated with anti-TG2 antibody as described above. The bound radiolabeled GTP was visualized by autoradiography, following SDS–PAGE (10% gel).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from Hemin/tRA induced K562 cells using the Trizol reagent (Invitrogen, Life Technologies, USA). One microgram of RNA was subjected to reverse transcription with the random nanomers and Takara RNA PCR kit (Takara Shuzo, Shiba, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers:

glycophorin A (416 bp); 5′-GTCAGCAATTG2TG2AGCATATCAGCA-3′ (sense); 5′-GATCACTTG2TCTCTG2CATTTTCTAT (antisense)
γ-globin (423 bp); 5′-GGACAAGGCTACTATCACAA-3′ (sense); 5′-CAGTG2GTATCTG2GAGGACAG-3′ (antisense)
CD36 (266 bp); 5′-CTG2GCTG2TG2TTTG2GAGGTATTCT-3′ (sense); 5′-AGCGTCCTG2GGTTACATTTTCC-3′ (antisense)
CD41b (379 bp); 5′-AGGCCTCTG2TCCAGCTAC-3′ (sense); 5′-GCCATTCCAGCCTCCGTG2-3′ (antisense)
β-actin (247 bp); 5′-CAAGAGATG2GCCACGGCTG2CT-3′ (sense); 5′-TCCTTCTG2CATCCTG2TCGGCA-3′ (antisense)

The PCR products were separated on 2% agarose gel electrophoresis containing ethidium bromide with 1× TAE buffer. After electrophoresis, the intensity of the bands obtained from RT-PCR result was estimated using TotalLab software of Frog Gel Image Analysis System (CorebioSystem Co., Seoul, Korea).

2.7. Assay of erythroid differentiation

K562 cells were incubated with Hemin/tRA in various time points indicated in the figure legends. Erythroid differentiation of K562 cells was scored by benzidine staining according to the procedure reported by Cooper et al. [27]. Benzidine positive cells (blue) were quantified ($n = 100$) by light microscopy.

Hemoglobinization was determined by the visible absorbance spectrum of the supernatant at 414 nm [28].

3. Results

3.1. Membrane recruitment of TG2 during the erythroid differentiation of K562 cells

To investigate whether TG2 might be involved in erythroid differentiation, the protein was separated into total, cytosol, and membrane fraction. It is worth to note that no increase of total TG2 level could be detected in the response to Hemin/tRA, Hemin alone, and PMA (Fig. 1A–C, upper panel). Membrane-bound TG2 appeared to be after 3 days in the treatment of Hemin/tRA (Fig. 1A, lower panel), but there was no increase of membrane bound TG2 in the response to Hemin during the experimental time period (Fig. 1B, lower panel). Furthermore, to investigate whether membrane bound TG2 could be increased during megakaryocytic differentiation of K562 cells, the cells were treated with PMA (10 nM) for the experimental time period. However, there was no increase of membrane bound TG2 in the response to PMA (Fig. 1C, lower panel), indicating that membrane recruitment of TG2 might be unique phenomenon during the erythroid differentiation of the cells induced by tRA.

3.2. Enhancement of membrane recruitment and GTP- photoaffinity by TG2 overexpression

To better understand the mechanism involved in the erythroid differentiation of K562 cell mediated by TG2, TG2 cDNA were stably transfected into K562 cells. Overexpression of TG2 was confirmed by Western blot (Fig. 2A, upper panel, time zero). TG2 transfected cells showed increase of the membrane bound TG2 level than vector transfected cells without Hemin/tRA (time zero) as well as after 2 days treatment, however, the membrane bound TG2 level in vector transfected cells was similar to that in TG2 transfected cells after 4 days (Fig. 2A, lower panel). Cytosolic TG2 level after fractionation was shown in Fig. 2A, middle panel.

To determine the ability of membrane TG2 to bind GTP, [α-^{32}P]GTP-photoaffinity analysis was performed. Each cell lines showed increase of GTP-photoaffinity of TG2 after 4 days in

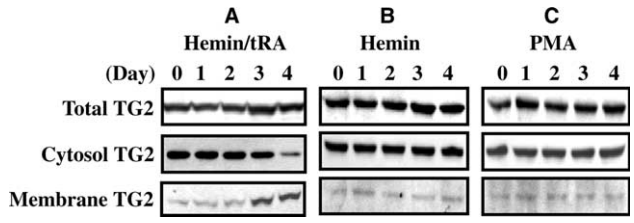

Fig. 1. Increase of membrane recruitment of TG2 during the erythroid differentiation of K562 cells. Each cell was incubated with Hemin/tRA, Hemin alone, and PMA for the time courses described in the figure. Proteins (50 μg) of the whole cell extracts, cytosol, and membrane fraction were isolated and subjected to 10% SDS–PAGE as described in Section 2. The expression levels of TG2 were determined via Western blot analysis.

the response of Hemin/tRA (Fig. 2B). When GTP-photoaffinity of TG2 was measured in densitometry (Fig. 2C), vector transfected cells after 4 days showed 2.3-fold increase than those cells after 2 days. Furthermore, TG2 transfected cells revealed 2.5- and 4.2-fold after 2 and 4 days, respectively, compared to vector transfected cells after 2 days. Therefore, these results indicated that the ability of membrane TG2 to bind GTP was also increased during the erythroid differentiation of K562 cell.

3.3. Activation of PI3K/Akt signaling pathway and inactivation of ERK1/2 phosphorylation by TG2 overexpression

To investigate which signaling proteins might be involved in erythroid differentiation by overexpression of TG2, Western blot was performed using specific antibodies that recognize only the phosphorylated forms. TG2-transfected cells showed activation of Akt phosphorylation in the response to Hemin/tRA for 2 days (Fig. 3A). However, there was no activation of p38 MAPK and JNK, indicating that overexpression of TG2 regulated PI3K/Akt signaling during the erythroid differentiation. On the other hand, ERK1/2 phosphorylation was decreased in TG2 transfected cells. Previous investigations [29,30] also showed that inhibition of ERK is involved in erythroid differentiation.

To further investigate downstream activation of PI3K/Akt signaling by overexpression of TG2, cyclic AMP-response element-binding protein (CREB) activation was measured. TG2 transfected cells showed increase of CREB phosphorylation compared with vector transfected cells (Fig. 3B). However, no increase of its phosphorylation could be detected in the presence of Wortmannin, which is a specific inhibitor of PI3K.

Erythroid differentiation of K562 cells was also confirmed by RT-PCR analysis with several different erythroid lineage marker genes. The mRNAs encoding γ-globin and CD36 in TG2 transfected cells clearly increased (Fig. 3C), but no change in their mRNAs in the presence of the inhibitor was observed.

3.4. Acceleration of erythroid differentiation of K562 cells by TG2 overexpression

To confirm biochemical studies above, benzidine staining on phase contrast microscopy was performed. Benzidine positive cells were clearly increased in TG2 transfected cells incubated

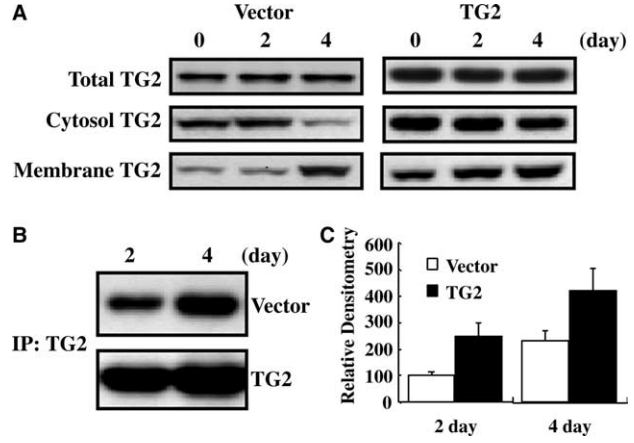

Fig. 2. Overexpression of TG2 increases TG2 recruitment into membrane and the ability of GTP-photoaffinity of TG2. (A) The cells were transfected with pCDNA-TG2 cDNA. Expression of TG2 (approximately 87 kDa) was analyzed by Western blot using a specific antibody to TG2. The cells were incubated with Hemin/tRA for the time period indicated in the figure. Proteins (50 μg) of the whole cell extracts, cytosol, and membrane fraction were isolated and subjected to 10% SDS–PAGE as described in Section 2. The TG2 levels were determined via Western blot analysis. (B) GTP binding activity of membrane bound TG2 was determined using affinity labeling with radioactive GTP as described in Section 2. [α-^{32}P]GTP-bound TG2 was then immunoprecipitated with anti-TG2 antibody. The bound radiolabeled GTP was visualized by autoradiography, following SDS–PAGE (10% gel). (C) Radiolabeled GTP was quantitated by densitometry. Values are means for 3 independent experiments.

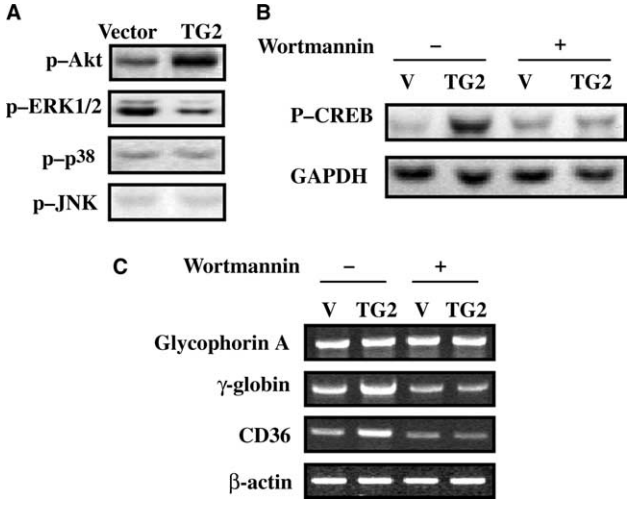

Fig. 3. Overexpression of TG2 increases activation of PI3K/Akt signaling pathway as well as the erythroid lineage marker genes. (A) Proteins (25 μg) were subjected to 10% SDS–PAGE. Phospho-specific antibody was used to measure the activation of Akt in the response of Hemin/tRA for 2 days. The blots were stripped and then reprobed with anti-p-ERK1/2, anti-p-p38 MAPK, and anti-p-JNK. (B) The cells were incubated with Hemin/tRA for 2 days in the absence or in the presence of Wortmannin (200 nM), PI3K specific inhibitor. Western blot analysis was performed using anti-phospho-CREB antibody on 10% SDS–PAGE. GAPDH indicated that equal amounts of proteins were loaded in each lane (low panel). (C) One μg of total RNA from each cell line was subjected to RT-PCR using primers specifically designed for several erythroid lineage marker genes as described in Section 2. β-Actin mRNA expression indicated that equal amounts of mRNA were used for RT-PCR in each lane (lowest panel).

with Hemin/tRA for 2 days and even more cells after 4 days (Fig. 4A). Indeed, when the cells were counted ($n = 100$), erythroid differentiated cells appeared to be 17% and 65% in TG2 transfected cells, but 2% and 14% in vector transfected cells after 2 and 4 days, respectively (Fig. 4B). Hemoglobinization after treatment of Hemin/tRA for 2 days by the visible absorbance spectrum of the supernatant at 414 nm was also increased in TG2 transfected cells (Fig. 4C).

Because hemin is known to induce erythroid differentiation of K562 cells reversibly [22], the K562 cells were treated with Hemin for 4 days and further incubated without hemin for 4 days and then performed benzidine staining on phase contrast microscopy. TG2 transfected cells showed longer differentiation state after removal of hemin than vector transfected cells (data not shown), indicating that overexpression of TG2 might lead to irreversibility of hemin-induced differentiation.

3.5. *Adrenergic receptor mediated signaling by overexpression of TG2 involved in the erythroid differentiation of K562 cells*

Because it is well known that TG2 is involved in adrenergic receptor mediated signaling (4), the cells were treated with Epinephrine (10^{-5} M) for 2 days and performed for RT-PCR. The mRNAs encoding γ-globin and CD36 clearly increased in TG2 transfected cells, but CD41b, one of megakaryocytic marker gene, had no change in its mRNA (Fig. 5), suggesting that adrenergic receptor mediated signaling by overexpression of TG2 might also be involved in the erythroid differentiation of K562 cells.

4. Discussion

TG2 is a soluble protein, mainly localized in the cytosolic cell compartment, with a small fraction of the enzyme in the membrane and extracellular matrix. The externalization of this enzyme from cells is tightly regulated by a number of factors, and is needed to stabilize the extracellular matrix, and to facilitate cell adhesion and motility [21,31]. Overexpression of

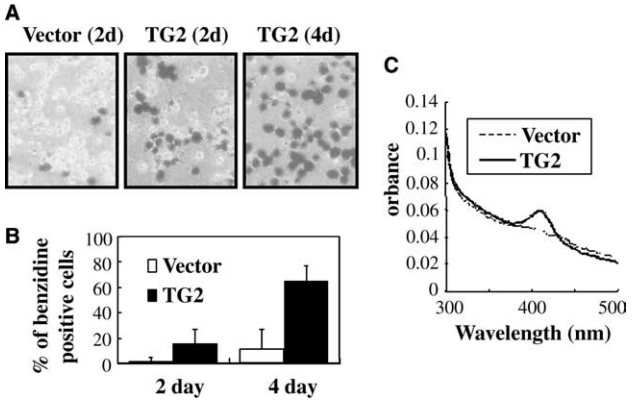

Fig. 4. Acceleration of the erythroid differentiation of K562 cells by TG2 overexpression. (A) The cells were incubated with Hemin/tRA for 2 and 4 days and performed benzidine staining as described in Section 2. (B) Benzidine positive cells were counted on phase contrast microscopy per 100 cells. (C) Hemoglobinization of these cells after treatment of Hemin/tRA for 2 days was determined by the visible absorbance spectrum of the supernatant as described in Section 2.

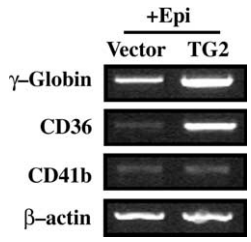

Fig. 5. Adrenergic receptor mediated signaling by TG2 involved in the erythroid differentiation of K562 cells. Each cell line was incubated with Epinephrine (10^{-5} M) for 2 days. One μg of total RNA from each cell line was subjected to RT-PCR using primers specifically designed for several erythroid lineage marker genes as described in Section 2. CD41b is one of megakaryocytic differentiation marker genes. β-Actin mRNA expression was also examined as a control (lowest panel).

TG2 involved in many patho-physiological conditions can be associated with the translocation of TG2 to membrane compartment or in extracellular matrix [21]. However, in the present study, membrane recruitment of TG2 plays an important role in erythroid differentiation of K562 cells and overexpression of TG2 accelerates erythroid differentiation of K562 cells via activation of PI3K/Akt signaling pathway.

It is well known that all tRA is the most powerful inducer of TG2 expression via nuclear receptors [21,32,33]. However, Western blot shown in Fig. 1 of the present paper demonstrates that, after three days of treatment of Hemin/tRA, the total TG2 level did not change in K562 cells, which is consistent result with previous study [23]. Possible explanations for this may be as follows; (1) the low expression level of either RARs or RXRs resulting in not enough heterodimerization of these receptors to induce TG2 expression or (2) these cell lines may have a different promoter region of TG2, which could not be induced in the response to tRA. In these regards, the latter is more likely in K562 cells, because we have found tRA-induced gene expression of ganglioside GD3 synthase that increases localization of TG2 into the membrane during the erythroid differentiation of the cells in the response to Hemin/tRA (Manuscript preparation). Grignani et al. [34] reported that expression of either RAR alpha or promyelocytic leukemia-specific PML-RAR alpha protein in K562 cells resulted in a reduced expression of erythroid differentiation markers and a reduced sensitivity to the erythroid differentiative action of heme. Unfortunately, the functions of TG2 as well as tRA effects on their study are not clear. On the other hand, expression of the PML-RAR alpha gene in myeloid U937 precursor cells resulted in the ability of these cells to induce TG2 in response to tRA during the granulocytic differentiation of these cells [35], suggesting that stimulating effect of retinoids on the expression of TG2 is different depending on the cell type. In addition, there is no increased TG2 expression even if the cells were treated with Hemin/tRA and delayed differentiation in vector transfected cells than in TG2 transfected cells, suggesting that suppression of TG2 expression may retard the erythroid differentiation of K562 cells. Therefore, our study may give a new insight for another aspect of the development of this disease and provide a rationale for therapy with agents that increase expression of TG2 that deserves further investigation.

The signaling pathway of PI3K/Akt is involved in regulating differentiation in many cell types [36,37]. The protein kinase Akt is one of the targets of PI3K and plays an important role in the regulation of cell death and survival [38,39]. Our study also showed that overexpression of TG2 leads to phosphorylation of Akt in Ser-473 in the response of Hemin/tRA as well as the acceleration of erythroid differentiation of K562 cells, because inhibition of PI3K by its specific inhibitor resulted in a block of differentiation, according to biochemical criteria. In addition, the cyclic AMP-response element-binding protein (CREB), one of Akt substrates [40], is a transcription factor that is target of a variety of signaling pathways mediating cell responses to extracellular stimuli, involving proliferation, differentiation, and adaptive responses of cell process [41,42]. Our result that phosphorylation of CREB, one of the Akt substrates, was increased in TG2-transfected cells supports previous study that TG2 modulates the activation of CREB during neuronal differentiation [43]. Indeed, transcription levels of γ-globin [44] and CD36 [45] genes are regulated by CREB activation. Therefore, the coupling between cell differentiation and survival elicited by TG2 overexpression may result in allowing survival of the newly differentiated cell for a limited time period.

GTP-bound TG2 participates in signaling pathways that link cell surface receptors to intracellular effectors because PI3K can also be stimulated by G protein-coupled receptors and integrin-dependent cell adhesion [46]. The most studied example of this involved the upregulation of phospholipase C activity by the α_1-adrenergic receptor (AR) [4,47,48], where it was shown that the α_1-AR stimulated the GTP binding activity of TG2. Moreover, it has been recently shown that TG2 has an integrin-binding ability and involves in adhesion and migration on fibronectin [49]. The ability of TG2 to transduce signals adds another dimension to its functionality and may help explain how the same protein can be linked to several, sometimes opposing, cellular responses. This point is exemplified by the finding [50] that a transamidation-defective form of TG2 as well as tRA induced TG2 expression protected cells from apoptosis, indicating that the GTP binding activity of TG2 likely mediated the survival advantage. Therefore, these investigations support our study that GTP bound TG2 may display the unique characteristic of being selectively recruited to the membrane, especially during the erythroid differentiation of K562 cells, which may facilitate receptor–receptor interactions and enhance ligand-independent or dependent signaling pathway.

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